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14. ABSTRACT
Post-chemotherapy cognitive impairment ("chemobrain") has been most often associated and studied in patients who have received adjuvant treatment for breast cancer. It is clear that chemotherapeutic drugs can produce cognitive deficits in humans; however, the mechanism is not known. Neurogenesis, the formation of new nerve cells, occurs throughout adulthood and is under the control of cell cycle regulators. Because some chemotherapeutic agents act by inhibiting cell cycle progression, we hypothesize that some of these agents produce cognitive impairment by disrupting neurogenesis in the hippocampus. The experiments in this Concept grant began to develop and utilize an animal model to study the effects of chemotherapeutic drugs, such as methotrexate, on cognitive function and neurogenesis. Mice were treated with saline or chemotherapeutic drugs. Behavioral testing was carried out and hippocampal neurogenesis was assessed. Our results show that methotrexate impairs cognitive function and reduces hippocampal neurogenesis in support of our hypothesis. Our results set the groundwork for future studies that use our animal model to test and develop therapeutic strategies to prevent and/or treat cognitive deficit in patients who undergo cancer chemotherapy.

15. SUBJECT TERMS
Hippocampal neurogenesis, chemotherapeutic drugs, cognitive deficit, chemobrain, cancer

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INTRODUCTION

Post-chemotherapy cognitive impairment has been most often associated and studied in patients who have received adjuvant treatment for breast cancer (van Dam et al., 1998; Inagaki et al., 2006; Furguson et al., 2007; Silverman et al., 2007; Kreukels et al., 2008). In the hippocampus, neural stem cells and progenitors proliferate and differentiate into neurons throughout adulthood (Abrous et al., 2005; Gould, 2007; Zhao et al., 2008). This phenomenon, called neurogenesis, is under the control of cell cycle regulators. Because some chemotherapeutic agents act by inhibiting cell cycle progression, our hypothesis is that some chemotherapeutic agents produce cognitive impairment by disrupting hippocampal neurogenesis. The experiments conducted in this Concept grant began to develop and utilize an animal model to study the effects of chemotherapeutic drugs on cognitive function and hippocampal neurogenesis. The first specific aim was to define the effects of these drugs on behavior. The second specific aim was to determine whether they disrupt hippocampal neurogenesis. The third specific aim was to ascertain if the behavioral deficits and inhibition of neurogenesis are decreased or prevented by drugs that stimulate neurogenesis. Mice were treated with saline or chemotherapeutic drugs. Neurological function was assessed and behavioral testing was carried out, and hippocampal neurogenesis was measured in brain sections by labeling cells with bromodeoxyuridine (BrdU) as well as by using other markers of neuronal proliferation.

BODY

Specific Aim 1: Define the effects of chemotherapeutic drugs on behavior.

<u>Task 1. Dose-response study using single drugs (months 1-4)</u>
Female mice are ordered at 5 weeks of age. At 7 weeks of age, the mice are injected i.p. with saline (0.9%), cyclophosphamide (10 mg/kg, 30 mg/kg or 100 mg/kg), methotrexate (3 mg/kg, 10 mg/kg or 30 mg/kg) or 5-fluorouracil (10 mg/kg, 30 mg/kg or 100 mg/kg). The mice are injected again one week and two weeks following the first injection for a total of three injections. One week after the last injection the mice underwent behavioral testing according to the following schedule:

Day 1: Open Field Test, Neuroscreen
Day 2: Spontaneous Alternation Test
Day 3: Fear Conditioning (Acquisition)
Day 4: Fear Conditioning (Context)
Day 5: Fear Conditioning (Cue)

We found toxicity after treatment with 5-fluorouracil. Some subjects died after the highest dose (100 mg/kg), and additional mice had to be treated in order to obtain a sufficient number of subjects in this treatment group. Although neuroscreen indicated that the cyclophosphamide- and methotrexate-treated subjects were normal, some of the mice that survived the highest dose of 5-fluorouracil (100 mg/kg) showed neurological deficits; they exhibited impairment of balance and locomotion (deficits in myorelaxation) and they were poorly groomed. Compared to saline-treated controls, treatment with cyclophosphamide or methotrexate did not affect locomotor activity (Fig. 1); however, treatment with 5-fluorouracil produced a statistically significant reduction in locomotor activity (Fig. 1). These results indicate that the highest dose of 5-fluorouracil produced nonspecific toxic effects, whereas cyclophosphamide and methotrexate did not produce overt toxicity.

Spontaneous alternation is a test of spatial working memory and is thought to reflect hippocampal-dependent processes (Gerlai, 2001). Although treatment with cyclophosphamide had no effect, treatment with the highest dose of methotrexate (30 mg/kg) and the highest dose of 5-fluorouracil (100 mg/kg) produced statistically significant deficits in spontaneous alternation (Fig. 2). As described about, we believe that the effects of 5-fluorouracil were due to nonspecific toxicity.

However, methotrexate did not affect either the neuroscreen or locomotor activity. Therefore, the deficit in performance in the spontaneous alternation tasks might reflect a specific effect on cognitive performance.

Fear conditioning is a form of learning in which fear (i.e., freezing behavior) is associated with a particular neutral context (e.g., a testing box) or neutral cue (e.g., a tone). This is accomplished by pairing a neutral stimulus with an aversive stimulus (e.g., footshock). Eventually, the neutral stimulus alone elicits freezing behavior. Context-specific fear conditioning is thought to involve the hippocampus, whereas cue-specific fear conditioning is thought to involve the amygdala (Anagnostaras et al., 2001; Gerlai, 2001; Maren, 2008). None of the drug treatments affected either context- or cue-specific fear conditioning (data not shown).

The grant proposal focused on using spontaneous alternation and fear conditioning as the two methods to assess the effects of drug treatment on memory and learning. Because a primary goal of the project was to develop an animal model to study the effects of chemotherapeutic drugs on cognitive function, we decided to evaluate two additional behavioral tests: the Barnes maze; and the passive avoidance test. The Barnes maze assesses spatial memory and is another method of assessing hippocampal-dependent learning (Barnes 1988; Holmes et al., 2002). Passive avoidance is fear-motivated test classically used to assess short- or long-term memory (Sarter et al., 1992; Deacon et al., 2002).

The Barnes maze consists of a 36 inch flat white circular platform with 20 equally spaced holes around the diameter. A bright light illuminates the maze. One of the 20 holes leads to a hide box where the mouse can escape, whereas the other 19 are false boxes that cannot be entered. Each of the holes has a separate photobeam and a light sensor. Breaking the photobeam at each of the 19 false boxes prior to entering the correct hide box is counted as an incorrect entry. The total number of incorrect entries is tallied for each trial. Latency is defined as the time it takes to enter the hide box. In the Training Phase, the mouse explores the surface area until it enters the hide box. The location of the hide box remains the same for each trial but is shifted across subjects to reduce the potential for unintended intra-maze cues. Three trials are conducted each day for 4 consecutive days. The Retention Phase measures the retention of spatial memory following a delay. After the completion of the 4 day training phase, two days go by without testing, then the subjects are retested for one day. The Reversal Phase measures performance on a memory reversal task. On the day following the retention phase, the hide box is relocated opposite (180 degrees) to its original placement. Three training trials are conducted each day for 2 consecutive days. The total time for testing is 9 days. Methotrexate (30 mg/kg) tended to increase the number of errors (i.e., incorrect entries) during acquisition and retention testing (Figure 3). The differences that occurred during acquisition and retention testing approached statistical significance; however, statistical power analysis indicated that the sample size was too low. However, methotrexate produced a statistically significant increase in errors during reversal testing. These results indicate that methotrexate impaired performance in the Barnes maze.

The passive avoidance task is a fear-motivated avoidance task in which the mouse learns to refrain from stepping through a door to an apparently safer but previously punished dark compartment. The latency to refrain from crossing into the punished compartment serves as an index of the ability to avoid, and allows memory of the previously punished response to be assessed. The training apparatus consists of a rectangular chamber divided into 2 compartments. One compartment is lighted by an overhead stimulus light, whereas the other compartment remains dark. The two compartments are separated by an automatic guillotine door, and each has a grid floor placed through which a footshock can be delivered. For habituation, the mouse is placed in the lighted compartment, facing away from the dark compartment and allowed to explore for 30 seconds. After 30 seconds the door is raised and the mouse is allowed to enter the other compartment. When the mouse enters the dark compartment with all four paws, the guillotine door is closed, and the latency to enter (from the time the door is lifted) is recorded. Once the mouse crosses to the darkened chamber the door closed and the mouse is immediately removed and returned to the home cage. Training takes place one hour after the completion of habituation. The mouse is placed in the lighted compartment and allowed to explore for 30 seconds, after which the guillotine door is lifted. When the mouse enters the dark compartment with all four paws, the guillotine door is closed, and the latency to enter is recorded. Three seconds after the door is closed a footshock (0.7 mA, 2 seconds duration) is delivered. The mouse is immediately removed and returned to the home cage. Testing occurs 24 hours and 7 days after the training session. On the test day the mouse is returned to the lighted compartment and after 30 seconds the guillotine door is lifted. When the mouse enters the dark compartment with all four paws, the guillotine door is closed, and the latency to enter the dark compartment is recorded. The mouse is removed and returned to the homecage. None of the drug treatments the latency to enter the dark compartment when measured 24 hours or 7 days post-training (data not shown).

In summary, 5-fluorouracil reduced performance in one test of cognitive function (i.e., spontaneous alternation), but this was probably due to drug-induced toxicity. However, methotrexate (30 mg/kg) produced deficits in two tests of cognitive function: the spontaneous alternation test; and the Barnes maze. Treatment with methotrexate did not produce changes in the neuroscreen or in locomotor activity, indicating that the decreased performance in the cognitive tests was not due nonspecific behavioral deficits or performance impairing effects of the drug. Therefore, the chemotherapeutic drug methotrexate produced deficits in performance on tasks that assess memory in our animal model of post-chemotherapy cognitive impairment. Because these two tests involve hippocampal – dependent processes, the results suggest that hippocampal function might be disrupted by drug treatment. This was assessed in Specific Aim 2.

Task 2. Combination drug study (months 5-8)
We did not carry out this task because the design and analysis of this experiment was complicated by our finding that 5-fluorouracil produced unexpected toxicity. We would expect that toxicity would be increased if 5-fluorouracil was administered in combination with the two other drugs, and this would make any behavioral effects extremely difficult to interpret.

Specific Aim 2: Determine effects of chemotherapeutic drugs on neurogenesis in the hippocampus.
Task 3. Dose-response study using single drugs (months 1-4)
Another cohort of mice was used to assess neurogenesis. These mice did not undergo behavioral testing. Female mice are ordered at 5 weeks of age. At 7 weeks of age, the mice are injected i.p. with saline (0.9%), cyclophosphamide (10 mg/kg, 30 mg/kg or 100 mg/kg), methotrexate (3 mg/kg, 10 mg/kg or 30 mg/kg) or 5-fluorouracil (10 mg/kg, 30 mg/kg or 100 mg/kg). The mice are injected again one week and two weeks following the first injection. In order to measure proliferating cells, six days after the last injection the mice are treated with bromodeoxyuridine (BrdU; 50mg/kg/i.p.) every 2 hours for a total of 4 injections. Twenty-four hours after the first BrdU injection, the mice are sacrificed and the brains were stored in formalin prior to embedding in paraffin and sectioning.

We found that BrdU labeling was significantly reduced (38%) only in mice that received the highest dose of methotrexate (30 mg/kg; Figure 4). BrdU labels all mitotic cells, whereas doublecortin (DCX) is expressed almost exclusively in immature neurons (i.e., neuroblasts). In order to determine whether the reduction in BrdU labeling was in neurons, slides were double-stained for BrdU and DCX (Figure 5). Treatment with methotrexate reduced the number of cells that co-express BrdU and DCX.

This finding shows that the reduction in proliferation occurred primarily in neural cells, and reflects methotrexate-induced decreases in neurogenesis. We have carried out TUNEL staining (data not shown) and found that there was no increase in apoptosis (programmed cell death), supporting our hypothesis that the effects of methotrexate were limited to a reduction in neurogenesis and not due to generalized tissue toxicity. Glial fibrillary acidic protein (GFAP) is a marker for glia. Immunohistohemical and Western Blot analyses showed that methotrexate increased GFAP expression (Figure 6.). Therefore, methotrexate decreased neurogenesis, but triggered gliogenesis in the hippocampus.

In summary, methotrexate was found to impair hippocampal neurogenesis. The results of this experiment support our hypothesis that some chemotherapeutic drugs disrupt hippocampal neurogenesis. We did not expect that cyclophosphamide would affect either behavior or neurogenesis because it does not readily cross the blood-brain barrier (Genka et al., 1990). Although 5-fluorouracil produced toxic effects, this was not accompanied by changes in neurogenesis. Only methotrexate affected both tests of spatial memory and reduced hippocampal neurogenesis. These results demonstrate that the decreases the decreases in hippocampal neurogenesis produced by methotrexate are directly associated with deficits in tests that assess spatial and/or working memory and hippocampal function. Furthermore, these findings support the overall goal of this Concept grant; to begin to develop and utilize an animal model to study the effects of chemotherapeutic drugs on cognitive function and hippocampal neurogenesis.

Task 4. Combination drug study (months 5-8)
As described under Task 2, we did not carry out Task 5 because 5-fluorouracil produced unexpected toxicity.

Specific Aim 3: Test whether drugs that stimulate neurogenesis can prevent or decrease the behavioral deficits produced by chemotheraputic drugs.

Task 5. Effects of treatment on drug-induced behavioral deficits (months 9-12)
We were unable to initiate the experiments assessing pharmacotherapeutic approaches to treat the chemotherapeutic drug-induced behavioral deficits (Task 5) and reduction in neurogenesis (Task 6). There were two primary reasons for this. First, we had negative results in one of the two tests of cognitive function (i.e., fear conditioning). Because a primary goal of the project was to develop the animal model, we decided that it was important to devote time and effort to evaluate two additional behavioral tests: the Barnes maze; and the passive avoidance test. We believe that this was a good decision because we found positive effects in the Barnes maze. Second, we found that the sectioning of the brain tissue and the subsequent processing for the immunohistochemical analyses was very labor intensive and time consuming. We now realize that our initial goals and objectives

Task 6. Effects of treatment on neurogenesis (months 9-12)
Please see Task 5.

***List of Personnel Receiving Pay from the Research Effort*:**
Robert N. Pechnick, Ph.D. (Principal Investigator)
Vera Chesnokova, Ph.D. (Co-Investigator)
Kevin Reyes (Research Laboratory Associate).

KEY RESEARCH ACCOMPLISHMENTS

- Treatment with the chemotherapeutic drug methotrexate disrupted performance on the spontaneous alternation task and in the Barnes maze, but did not produce nonspecific deficits in behavior.

- The spontaneous alternation task and the Barnes maze are tests of spatial working memory and are thought to reflect hippocampal-dependent processes. Therefore, the data suggest that treatment with methotrexate disrupted hippocampal function.

- Treatment with methotrexate also reduced hippocampal neurogenesis. This was not due to generalized tissue toxicity.

- These results support our hypothesis that some chemotherapeutic agents produce cognitive impairment by disrupting hippocampal neurogenesis.

- The experiments have led to the development of an animal model to study the effects of chemotherapeutic drugs on cognitive function and hippocampal neurogenesis.

- This new model might drive the development of potential approaches to treat and or prevent post-chemotherapy cognitive impairment.

REPORTABLE OUTCOMES

Pechnick, R.N. Neurogenesis, Depression and Chemobrain. Department of Psychiatry Grand Rounds, Department of Psychiatry, Harbor/UCLA Medical Center, Torrance, California, November 2009.

Pechnick, R.N. Neurogenesis, Depression and Chemobrain, Department of Pharmacology, University of Texas Health Science Center, San Antonio, Texas, April 2010.

Pechnick, R.N., Reyes, K.C., Das, M, Lacayo, L.M., Farrokhi, C., Zonis, S. and Chesnokova V. Cognitive impairment and decreased hippocampal neurogenesis after treatment with chemotherapeutic drugs. Abstract presented at the Experimental Biology meeting, Anaheim, California, April 2010. (see Appendix)

Pechnick, R.N., Reyes, K.C., Das, M, Lacayo, L.M., Farrokhi, C., Zonis, S. and Chesnokova V. Cognitive impairment and decreased hippocampal neurogenesis after treatment with chemotherapeutic drugs. Poster presented at the Experimental Biology meeting, Anaheim, California, April 2010. (see Appendix)

Pechnick, R.N., Reyes, K.C., Das, M, Lacayo, L.M., Farrokhi, C., Zonis, S. and Chesnokova V. Animal Models, Cell Cycle Regulators, and Neurobehavioral Disorders. Presented at the annual meeting of the American Psychiatric Association, New Orleans, Louisiana, May 2010 (see Appendix).

Funding Applied For Based On Work Supported By This Award: Development of Treatment for Post-Chemotherapy Cognitive Impairment, Idea Award BC101415, submitted.

CONCLUSION

The results are very encouraging. It is clear that chemotherapeutic drugs produce cognitive deficits in humans; however, the mechanism is not known. Currently, it is not possible to measure neurogenesis in living humans, so data must be obtained from experimental animals. The development of appropriate and meaningful animal models is critical in order to understand the fundamental mechanisms and pathological processes that underlie post-chemotherapy cognitive impairment. Our results show that the chemotherapeutic drug methotrexate impairs cognitive function. Thus, our animal model might be useful to determine whether new chemotherapeutic drugs would be likely to produce cognitive deficits in humans. The finding that cyclophosphamide affected neither cognitive function nor neurogenesis suggests that perhaps not all chemotherapeutic drugs necessarily produce adverse cognitive effects in humans; it might be due to only one drug in the combination of chemotherapeutic drugs given to a patient. Our data also show that methotrexate reduces hippocampal neurogenesis. This supports our hypothesis that some chemotherapeutic agents produce cognitive impairment by disrupting hippocampal neurogenesis. Certain drugs have been shown to stimulate hippocampal neurogenesis. Based upon our findings, it is plausible that drugs that stimulate neurogenesis might be effective in treating post-chemotherapy cognitive impairment. The results of the project have set the groundwork for future studies that use our animal model to test and develop therapeutic strategies to prevent and/or treat the cognitive deficits in individuals who undergo cancer chemotherapy. Importantly, some of the drugs that are known to stimulate hippocampal neurogenesis already are used in clinical practice to treat other conditions. Thus, the pathway to begin to use these drugs to treat post-chemotherapy cognitive impairment might be short.

REFERENCES

Abrous, D.N., Koeh,l M., and Le Moal, M. 2005. Adult neurogenesis: from precursors to network and physiology *Physiol Rev* 85: 523-569.

Anagnostaras, S.G., Gale, G.D., and Fanselow, M.S. 2001. Hippocampus and contextual fear conditioning: recent controversies and advances. *Hippocampus* 11:8-17.

Barnes, C.A. 1988. Spatial learning and memory processes: the search for their neurobiological mechanisms in the rat. *Trends Neurosci* 11:163-169.

Deacon, R.M.J., Bannerman, D.M., Kirby, B.P., Croucher, A. and Rawlins, J.N.P. 2002. Effects of cytotoxic hippocampal lesions in mice on a cognitive test battery. *Behav Brain Res* 133:57-68.
Ferguson, R.J., McDonald, B.C., Saykin, A.J., and Ahles, T.A. 2007. Brain structure and function differences in monozygotic twins: possible effects of breast cancer chemotherapy *J Clin Oncol* 25:3866-3870.

Genka, S., Deutsch, J., Stahle, P.L., Shetty, U.H., John, V., Robinson, C., Rapaport, S.I., and Greig, N.H. 1990. Brain and plasma pharmacokinetics and anticancer activities of cyclophosphamide and phosphoramide mustard in the rat. *Cancer Chemother Pharmacol* 27:1-7.

Gerlai, R. 2001. Behavioral tests of hippocampal function: simple paradigms complex problems. *Behav Brain Res* 125:269-277.

Gould, E. 2007. How widespread is adult neurogenesis in mammals? *Nat Rev Neurosci* 8:481-488.

Holmes, A., Wrenn, C.C., Harris, A.P., Thayer K.E. and Crawley JN. 2002. Behavioral profiles of inbred strains on novel olfactory, spatial and emotional tests for reference memory in mice. *Genes Brain Behav* 1:55-69.

Inagaki, M., Yoshikawa, E., Matsuoka, Y., Sugawara, Y., Nakano, T., Akechi, T., Wada, N., Imoto, S., Murakami, K., and Uchitomi, Y. 2007. Smaller regional volumes of brain gray and white matter demonstrated in breast cancer survivors exposed to adjuvant chemotherapy. *Cancer* 109:146-156.

Kreukels, B.P.C., Hamburger, H.L., de Ruiter, M.B., van Dam, F.S., Ridderinkhof, K.R., Boogerd, W, and Schagen, S.B. 2008. ERP amplitude and latency in breast cancer survivors treated with adjuvant chemotherapy. *Clin Neurophysiol* 119:533-541.

Maren, S. 2008. Pavlovian fear conditioning as a behavioral assay for hippocampus and amygdala function: cautions and caveats. *Eur J Neurosci* 28:1661-1666.

Sarter M., Hagan J. and Dudchenko, P. 1992. Behavioral screening for cognition enhancers: from indiscriminate to valid testing. Part II. *Psychopharmacology* 107:461-473.

Silverman, D.H., Dy, C.J., Castellon, S.A., Lai, J., Pio, B.S., Abraham, L., Waddell, K., Petersen, L., Phelps, M.E., and Ganz, P.A. 2007. Altered frontocortical, cerebellar, and basal ganglia activity in adjuvant-treated breast cancer survivors 5-10 years after chemotherapy. *Breast Cancer Res Treat* 103:303-311.

van Dam, F.S., Schagen, S.B., Muller, M.J., Boogerd, W., vd Wall, E., Droogleever Fortuyn, M.E., and Rodenhuis, S. 1998. Impairment of cognitive function in women receiving adjuvant treatment for high-risk breast cancer: high-dose versus standard-dose chemotherapy. *J Natl Cancer Inst* 90:210-218.

Zhao, C., Deng, W., and Gage, F.H. 2008. Mechanisms and functional implications of adult neurogenesis. *Cell* 132:645-60.

APPENDICES

Abstract, American Psychiatric Association, New Orleans, Louisiana, May 2010

Abstract, Experimental Biology meeting, Anaheim, California, April 2010.

Poster, Experimental Biology meeting, Anaheim, California, April 2010.

Presented at the annual meeting of the American Psychiatric Association, New Orleans, Louisiana, May 2010

Animal Models, Cell Cycle Regulators, and Neurobehavioral Disorders

Robert N. Pechnick, Kevin C. Reyes, Liliana M. Lacayo, Catherine Farrokhi, Svetlana Zonis and Vera Chesnokova.

In the hippocampus, neural stem cells and progenitors proliferate and differentiate into neurons throughout adulthood. The functional significance of adult neurogenesis is not known.

The hippocampus is involved in a number of important functions, including memory formation and retrieval, learning, and neuroendocrine and mood regulation. Many studies have attempted to link changes in hippocampal neurogenesis to alterations or deficits in these endpoints.

The relationships among hippocampal neurogenesis, depression and the mechanism of action of antidepressant drugs have generated a considerable amount of interest and controversy. Neurogenesis is under the control of cell cycle regulators. p21Cip1, a cyclin-dependent kinase inhibitor, restrains cell-cycle progression and proliferation throughout the body. It is found in neuroblasts and newly developing neurons in the subgranular zone of the hippocampus. Chronic treatment with the tricyclic antidepressant imipramine decreases p21Cip1 transcript and protein levels, stimulates neurogenesis in this region and produces antidepressant-like behavior in animal models. Moreover, mice lacking p21Cip1 have increased rates of hippocampal neurogenesis. Thus, p21Cip1 restrains neurogenesis in the hippocampus, and antidepressant-induced stimulation of neurogenesis might be due to decreased p21Cip1 expression. Cell-cycle regulation occurs downstream from the primary site of action of antidepressants, suggesting that new therapeutic strategies might directly target cell-cycle proteins.

Post-chemotherapy cognitive impairment, commonly called “chemobrain,” has long been recognized in cancer survivors. After cancer chemotherapy, patients frequently suffer from memory lapses, have trouble concentrating, are unable to remember details, and have problems doing more than one thing at a time (i.e., multitasking) and trouble remembering common words and names. Some chemotherapeutic agents, such as methotrexate, disrupt hippocampal neurogenesis and impair performance function in animal models of cognitive function. Therefore, post-chemotherapy-induced changes in neurogenesis might be a fundamental mechanism underlying the development. This information finding could lead to the development of treatment strategies to treat and/or prevent this frequent and troubling problem in cancer patients.

Abstract presented at the Experimental Biology meeting, Anaheim, California, April 2010.

Cognitive impairment and decreased hippocampal neurogenesis after treatment with chemotherapeutic drugs

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Post-chemotherapy cognitive impairment has long been recognized in cancer survivors; however, at the present time the cause(s) is not known. The objective of the present study was to determine the effects of chemotherapeutic drugs on cognitive function and hippocampal neurogenesis in the mouse. Adult female mice were injected once a week for 3 weeks with methotrexate (30 mg/kg/i.p.), cyclophosphamide (100 mg/kg/i.p.) or saline (0.9%/i.p.). One week after the last injection they underwent behavioral testing. Another cohort of mice did not undergo behavioral testing, but were treated with bromodeoxyuridine (BrdU; 50 mg/kg/i.p.; every 2 hr for a total of 4 injections) and sacrificed 24 hr later. There were no significant differences in locomotor activity among the treatment groups; however, spontaneous alternation was impaired in the methotrexate-treated subjects. BrdU incorporation was not affected in the cyclophosphamide-treated subjects, but was markedly reduced in the dentate gyrus of the hippocampus after treatment with methotrexate. These results suggest that post-chemotherapy cognitive impairment might be linked to drug-induced decreases in hippocampal neurogenesis. Supported by Department of Defense Breast Cancer Research Program grant number BC075629.

582.2

Cognitive Impairment and Decreased Hippocampal Neurogenesis after Treatment with Chemotherapeutic Drugs

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Introduction

Post-chemotherapy cognitive impairment, also known as "chemobrain," has been recognized in many cancer survivors. However, at the present time, the cause of this phenomenon is not known. The objective of the present study was to determine the effects of chemotherapeutic drugs on cognitive function and hippocampal neurogenesis in the mouse.

Methods

Animals
Seven week-old, female C57/BL6 mice were given intraperitoneal injections once a week for 3 weeks with either saline (0.9% NaCl) or one of the following chemotherapy doses: cyclophosphamide (CP) (10, 30, or 100 mg/kg), methotrexate (MTX) (3, 10, or 30 mg/kg), or 5-fluorouracil (5-FU) (10, 30, or 100 mg/kg). One week following the last injection, animals were used for behavioral testing or other experiments.

Behavioral Testing
One week after treatment, the animals tested using the following:

Open Field Test
Mice were placed in a 16" X 16" clear plexiglass box. Locomotor activity was automatically recorded for 60 minutes using PAS software (San Diego Instruments).

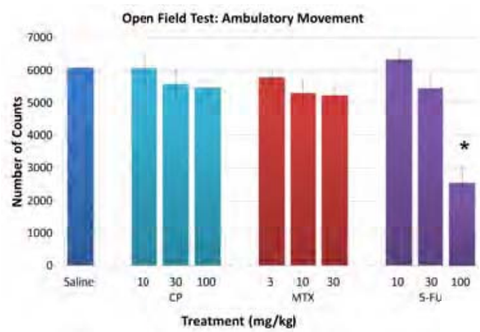
Spontaneous Alternation
Mice were placed in a plastic opaque Y-maze. Visiting the three different arms in succession was counted as a spontaneous alternation. The number of spontaneous alternations was divided by the number of alternations, given by number of arms visits minus 2, to determine the spontaneous alternation percentage. The sessions were recorded by an overhead mounted camera and manually scored by a blind observer.

Barnes Maze
Animals were placed on top of a brightly lit, 36" diameter, circular platform with 20 equally spaced holes along the outer edge. One of the 20 holes leads to a hide box while the other 19 lead to false boxes that cannot be entered. Animals were trained (acquisition) over 4 days (3 trials/day) to escape the surface and locate the hidebox. After a 2 day interim period, the mice were retested for latency and number of errors (retention). The test was followed by 2 days of reversal testing where the animals were required to find a new hidebox location (reversal).

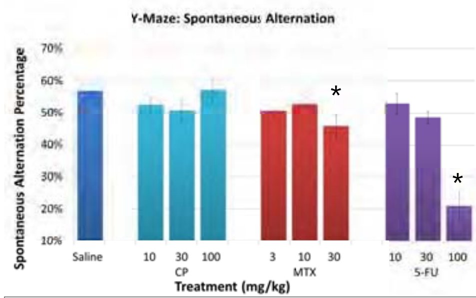
Neurogenesis
Six days after the last chemotherapy injection, mice that were not behaviorally tested were injected with bromodeoxyuridine (BrdU), a thymidine analogue and a standard for measuring neurogenesis, (50 mg/kg i.p. in 0.9% NaCl) 4 times every 2 hours. Twenty-four hours after the first BrdU injection, mice were euthanized and brains were collected.

One hemisphere of the brain was fixed in formaldehyde and embedded in paraffin. Five micron sagittal sections were cut and BrdU immunohistochemistry was performed. Every third section (of a total of 30 sections from 0.24 to 0.48mm lateral to midline) was counted blindly under a 200X objective. Cells were counted if they were within 2 cell diameters of the subgranular zone (SGZ) of the hippocampus. Certain sections were double-stained with BrdU and either doublecortin (DCX), a marker for developing neurons, or glial fibrillary acidic protein (GFAP), a marker for astrocytes. The antibodies were labeled with Alexa Fluor 568 (green) and Alexa Fluor 488 (red). Additionally, some hippocampi were dissected and western blots were performed for GFAP.

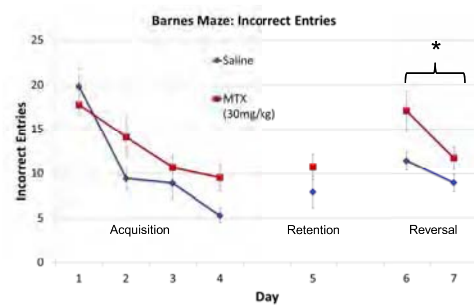
Results



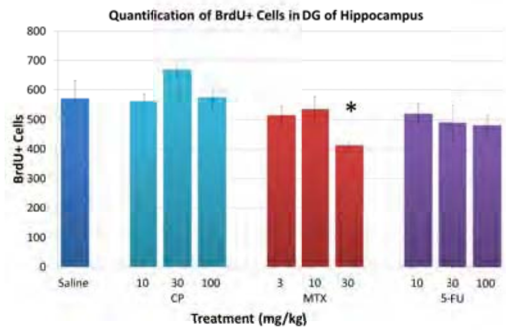
All treatments, excluding the high dose of 5-FU, did not alter locomotor activity. The high dose of 5-FU significantly reduced locomotor activity ($p<0.05$). Animals in this group exhibited sickness behavior.



MTX (30 mg/kg) significantly reduced spontaneous alternation activity. Although 5-FU (100 mg/kg) reduced spontaneous alternation as well, overall locomotor activity was drastically reduced.

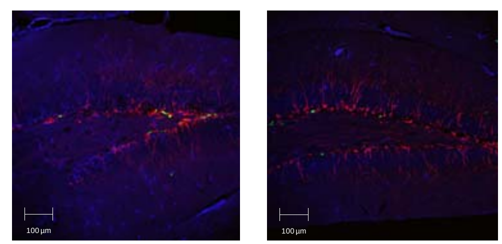


MTX (30 mg/kg) increased the incorrect entries made during reversal trials (Day 6-7).



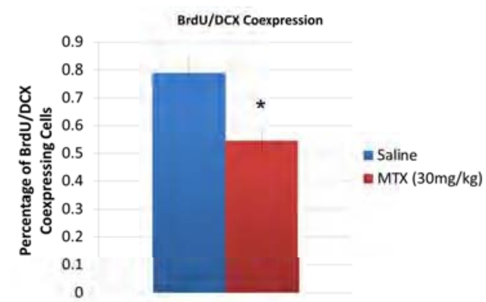
MTX (30 mg/kg) significantly reduced the number of BrdU+ cells in the dentate gyrus (DG) of the hippocampus.

Hippocampal BrdU/DCX Labeling



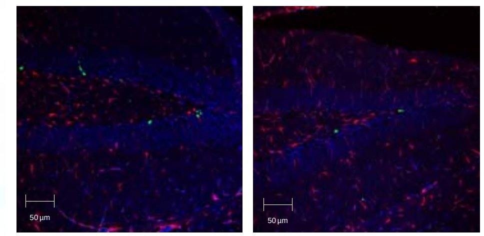
Saline *MTX (30 mg/kg)*

Confocal microscopy pictures were taken using 100X magnification. BrdU+ cells were visualized using Alexa Fluor 568 (green) while Alexa Fluor 568 (red) was used to visualize DCX expressing cells.



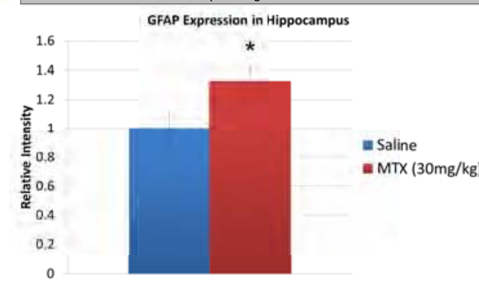
Treatment with MTX (30 mg/kg) reduced the amount of cells that both incorporated BrdU and expressed DCX.

Hippocampal BrdU/GFAP Labeling



Saline *MTX (30 mg/kg)*

Confocal microscopy pictures were taken using 200X magnification. BrdU+ cells were visualized using Alexa Fluor 568 (green) while Alexa Fluor 568 (red) was used to visualize GFAP expressing cells.



Animals treated with MTX (30 mg/kg) exhibited an increase in hippocampal GFAP expression relative to Saline controls. Quantification of western blots for GFAP was performed using NIH ImageJ software.

Conclusions

- Mice treated with MTX (30 mg/kg) exhibited reduced performance in behavioral tasks as demonstrated by the Y-maze and reversal trials of the Barnes Maze.
- Hippocampal neurogenesis is reduced in MTX (30 mg/kg) treated animals. More specifically, treatment with MTX (30 mg/kg) decreased the number of immature dividing neurons expressing DCX.
- Hippocampal GFAP expression increased in MTX (30 mg/kg) treated animals implying that a reduction in neurogenesis may be due to a shift from neuronal to glial lineage.
- A high dose of 5-FU (100 mg/kg) was highly toxic, resulting in a decrease in overall locomotor activity.
- The results of these experiments suggest the hippocampal neurogenesis may indeed be an important link between chemotherapy administration and cognitive impairment.

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SUPPORTING DATA

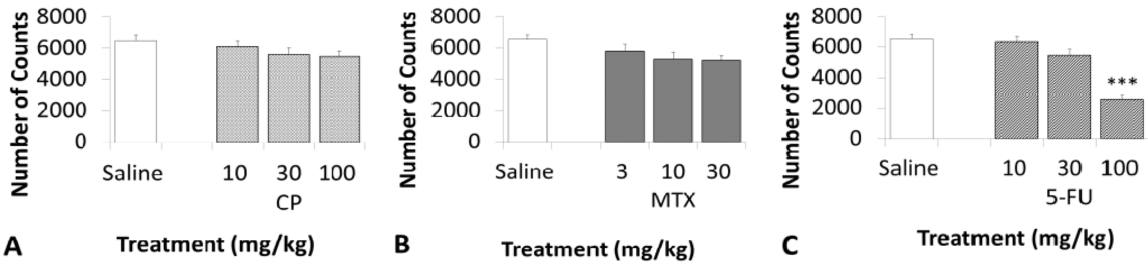

Figure 1. The effects of treatment with cyclophosphamide (CP; A), methotrexate (MTX; B) and 5-fluorouracil (5-FU; C) on locomotor activity in the open field test. Activity (number of beam breaks) was recorded and summed over 60 minutes. Cyclophosphamide and methotrexate had no effect, but the highest dose of 5-fluorouracil (100 mg/kg) significantly reduced locomotor activity. Values are expressed as the means + the standard errors of the mean (n=11-15 per group). ***P < .001 compared to saline-treated controls.

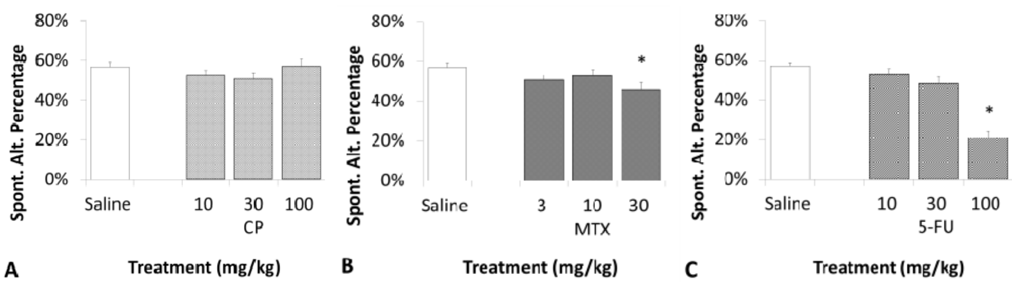

Figure 2. The effects of treatment with cyclophosphamide (CP; A), methotrexate (MTX; B) and 5-fluorouracil (5-FU; C) on spontaneous alternation. Cyclophosphamide had no effect, but the highest doses of methotrexate (30 mg/kg) and 5-fluorouracil (100 mg/kg) significantly reduced spontaneous alternation. Values are expressed as the means + the standard errors of the mean (n=10-15 per group). *P < .05 compared to saline-treated controls.

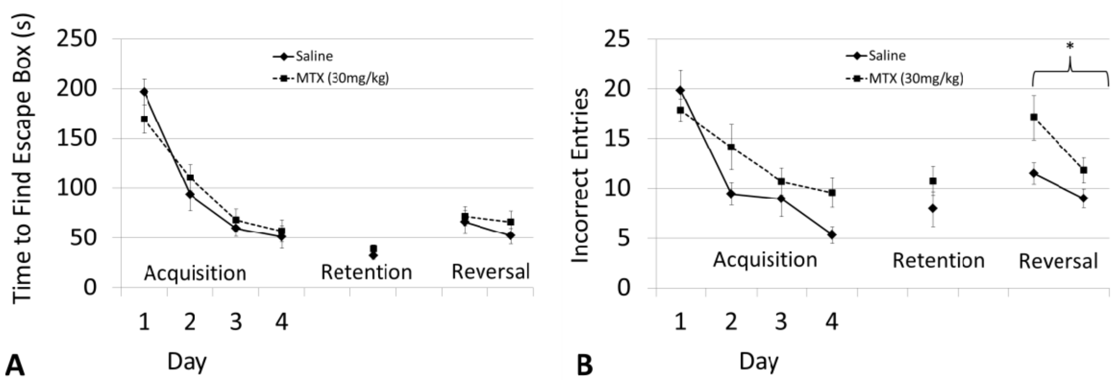

Figure 3. The effects of methotrexate (MTX; 30 mg/kg) on the time to escape (A) and the number of incorrect entries (i.e., errors; B) in the Barnes maze. Latency to enter the escape box was unaffected

by treatment (A), whereas methotrexate tended to increase the number of errors during acquisition and retention testing, and produced a statistically significant increase in errors during reversal testing (B). Values are expressed as the means + the standard errors of the mean (n=10-11 per group). *P < .05 compared to saline-treated controls.

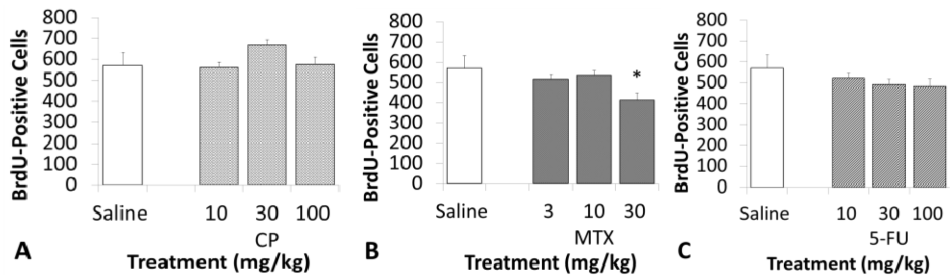

Figure 4. The effects of treatment with cyclophosphamide (CP; A), methotrexate (MTX; B) and 5-fluorouracil (5-FU; C) on BrdU incorporation in the dentate gyrus of the hippocampus. MTX (30 mg/kg) reduced the number BrdU-labeled cells. Values are expressed as the means + the standard errors of the mean (n=3 per group). *P < .05 compared to saline-treated controls.

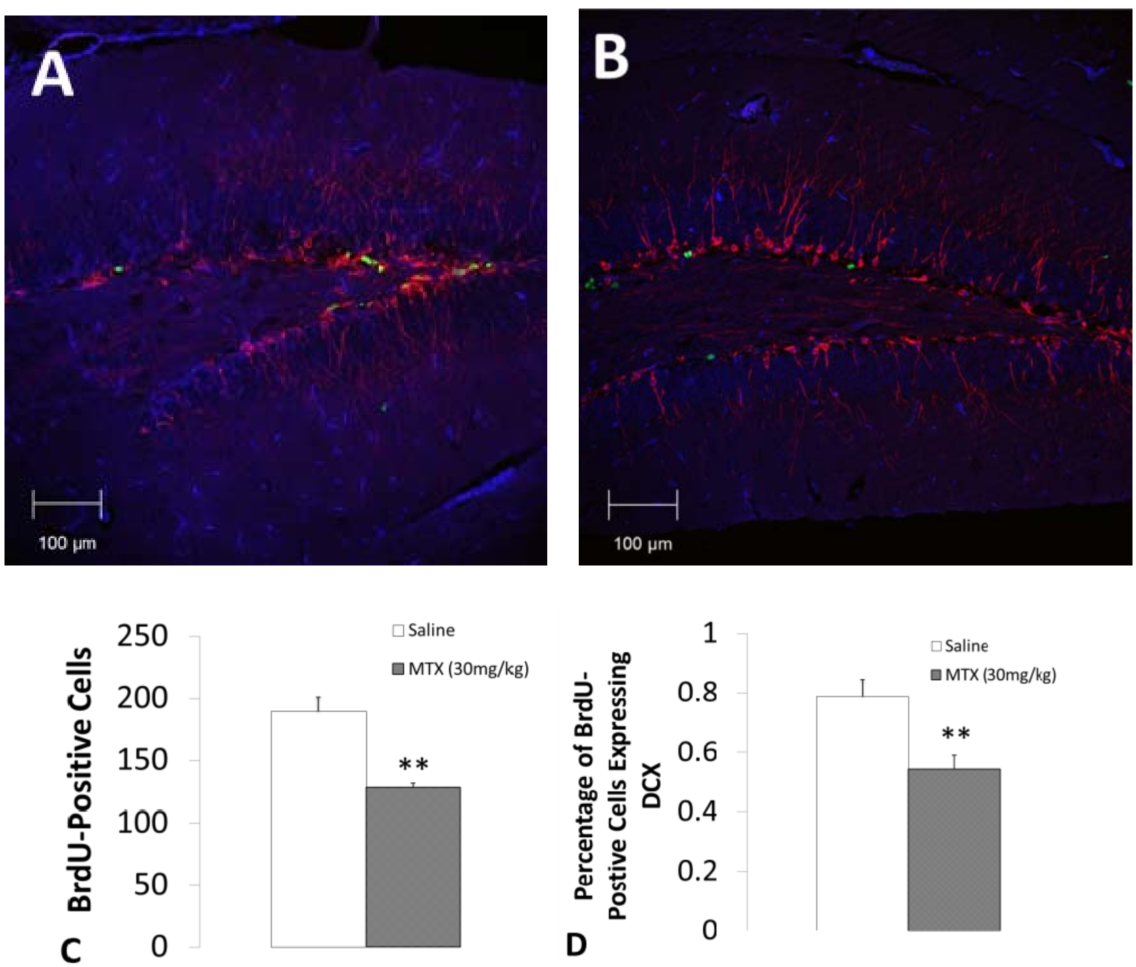

Figure 5. Hippocampal BrdU (green) and DCX (red) expression viewed at a 100X magnification in saline- (A) and methotrexate- (MTX; 30 mg/kg) treated mice (B). Treatment with methotrexate reduced the number of BrdU-positive cells in the dentate gyrus of the hippocampus (C) and decreased the percentage of BrdU-positive cells expressing DCX (D). Values (in C and D) are expressed as the means + the standard errors of the mean (n=4-5 per group). $^{**}P < .01$ compared to saline-treated controls.

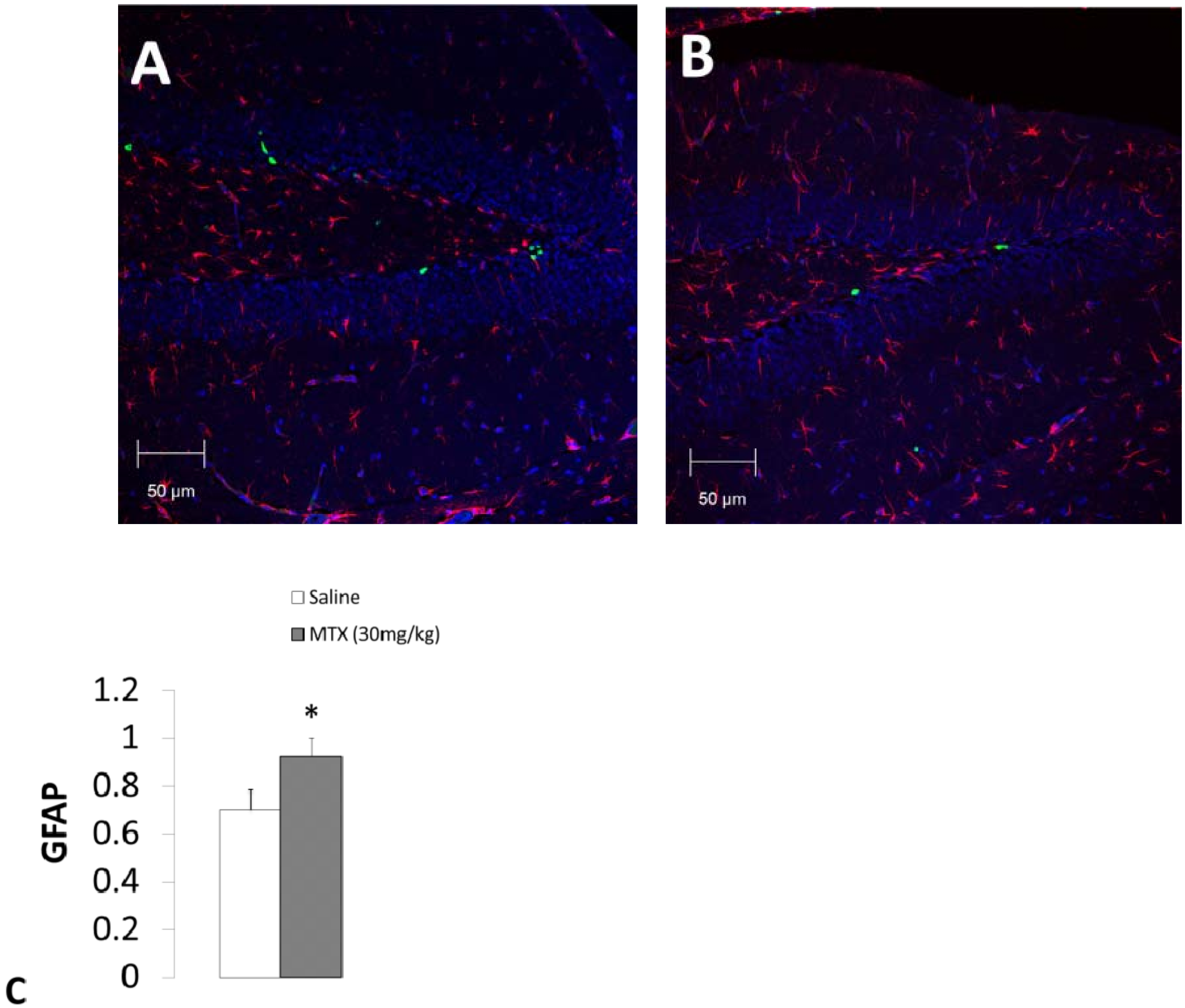

Figure 6. Hippocampal BrdU (green) and GFAP (red) expression viewed at a 200X magnification in saline- (A) and methotrexate- (MTX; 30 mg/kg) treated mice (B). Western Blot revealed increased levels of GFAP in the methotrexate- treated mice (C). Values (in C) are expressed as the means + the standard errors of the mean (n=4-5 per group). $^{*}P < .05$ compared to saline-treated controls.